EXHIBIT B

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Human inhibitor of apoptosis protein (IAP) *survivin* participates in regulation of chromosome segregation and mitotic exit

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ABSTRACT

A signaling cascade termed the "spindle checkpoint" monitors interactions between the kinetochores of chromosomes and spindle microtubules to prevent precocious separation of sister chromatids. We have investigated the role of human inhibitor of apoptosis protein (IAP) survivin in regulation of cell division. We demonstrate that HeLa and PtK1 cells transfected or microinjected with survivin anti-sense oligonucleotides produce significantly more polyploid and micronucleated progeny cells and show abortive mitosis when treated with spindle poisons. Furthermore, perturbation of survivin function in HeLa and PtK1 cells with anti-survivin antibodies at the beginning of mitosis affects the normal timing of separation of sister chromatids and disturbs the 3F3/2 phosphoepitope-recognized tension sensing mechanism of the spindle checkpoint. This leads to premature separation of sister chromatids, which results in an uneven distribution of chromosomes between the newly formed progeny cells—an event associated with tumor formation in many cell types. Finally, cells injected with anti-survivin antibody exit mitotic block induced with microtubule drugs. Our data suggest that survivin protein may function within the spindle checkpoint pathway.

Key words: survivin • mitosis • spindle checkpoint

Onset of anaphase normally occurs when all the chromosomes are aligned at the metaphase plate and equal physical tension created by the opposing microtubule pulling forces is applied to each pair of sister kinetochores. Misalignment of a chromosome(s) creates unequal tension at the kinetochores keeping the spindle checkpoint active (1, 2). If prolonged, this misalignment causes arrest of the cell cycle before sister chromatids separate (1–3). During the arrest, incorrectly aligned chromosome(s) are provided extra time to achieve bipolar attachments and to complete their movement to the spindle equator. Alignment of all the chromosomes switches off the checkpoint and activates a ubiquitin ligase, anaphase-promoting complex/cyclosome (APC/C), which selectively marks anaphase inhibitors for destruction (4, 5). Recently, proteins belonging to the family of inhibitors of apoptosis (IAPs) have been implicated

in the progression of cell cycle and in programmed cell death termed "apoptosis." The human IAP survivin appears to play a role in mitosis (M phase) and apoptosis, as cell populations with lower than normal levels of survivin mRNA or mutated survivin protein produce polyploid progeny cells and are sensitized to cell death (6–8). Moreover, mutant worms lacking the survivin-like *C. elegans* BIR-1 protein show defects in cytokinesis, which suggests a function for survivin and its homologues during late mitotic events (9, 10). BIR-1 physically interacts with the Aurora-like kinase AIR-2 and possibly affects chromosome behavior and organization of the midbody through this association (10). A recent knockout study shows that survivin is essential for early embryogenesis and that the protein transiently locates to the kinetochores of human cells from late prophase to metaphase (11, 12). Moreover, survivin has been shown to associate with the cyclin-dependent kinase $p34^{cdc2}$ and to be phosphorylated in M phase on Thr^{34} by $p34^{cdc2}$-cyclin B1 (13). Although it is clear from the data above that survivin and its homologies play a role during the M phase and in apoptosis, the actual signaling cascade in which survivin participates in is not known. Here we have investigated the effects of perturbing IAP survivin during M phase and provide evidence for a link between the control of mitotic progression and IAPs in mammalian cells.

MATERIALS AND METHODS

Cell culture

Adherent HeLa and Ptk1 (rat kangaroo) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Both cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Antibodies

Affinity-purified rabbit anti-human survivin antibody was custom manufactured for microinjection purposes by the Alpha Diagnostics International Inc. (San Antonio, TX). The antibody was diluted in KCl-PO_4 microinjection buffer at a stock solution concentration of 10 mg/ml. The needle concentration used in the microinjection experiments was 1–5 mg/ml. For immunolabeling, anti-3F3/2 ascites, a generous gift from Gary Gorbsky, were used at a concentration of 1:2500. In some experiments, Crest anti-centromere antibody (Cortex Biochem, San Leandro, CA) and anti-β-tubulin antibody (Sigma, St. Louis, MO) were used to label mitotic kinetochores and microtubules, respectively, at concentrations of 1:400 and 1:100.

Western blotting

Cycling and mitotic HeLa and PtK1 cell populations were lysed in RIPA buffer (20 mm Tris-HCL, pH 7.7; 100 mM KCl; 50 mM sucrose; 0.1 mM $CaCl_2$; 1 mM $MgCl_2$; 0.5% Triton X-100) with protease inhibitors (5 μg/ml leupeptin and pepstatin and 1 μg/ml aprotinin) for 12 min on ice with occasional vortexing. We centrifuged the extracts for 10 min at 15.000 *g* at 4°C and prepared supernatants in sample loading buffer with β-mercaptoethanol. Proteins were separated on a 10% sodium dodecylsulfate-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA). The membranes were blocked with 5% bovine serum albumin (BSA) in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 45 min,

washed, and incubated with polyclonal anti-survivin antibody (200 ng/ml in TBST) for 90 min. The washed membranes were incubated with HRP-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) 1:30,000 in TBST for 1 h. We visualized the proteins by using the Renaissance chemiluminescence kit (Dupond-NEN, Boston, MA).

Survivin antisense oligonucleotides and transfection experiments

Fluorescein isothiocyanate (FITC)-conjugated survivin antisense (7) (5'-TGTGCTATTCTGTGAATT) and a scrambled control (5'-TAAGCTGTTCTATGTGTT) 2'-O-methoxyethyl nucleoside containing (underlined in the sequence) oligonucleotides were synthesized with uniform phosphorothioate linkages. Oligonucleotides were administrated to cells by two methods: microinjection (see below) or liposome-mediated DNA transfer. HeLa and PtK1 cells were transfected with 200–400 nM concentrations of control or survivin antisense oligonucleotides using FuGENE 6 reagent (Roche Diagnostics Corporation, Indianapolis, IN), according to the manufacturer protocol. These concentrations have inhibited endogenous survivin RNA expression by at least 80% in HeLa cells (7). The transfected cells were incubated at 37°C for 16 to 24 h before harvest. Transfection efficiency typically varied between 55%–75%. In some experiments, cells growing on coverslips were transfected with the scrambled or survivin antisense oligonucleotides and were incubated for 12 hours at 37°C, after which nocodazole was added at concentration of 1.0 μg/ml. Cells on coverslips were harvested at 0-, 2-, 4-, 6-, and 8-h time points after addition of the drug.

Microinjection and live cell analysis

For live cell experiments and microinjection, we used a Narishige M-152 micromanipulator with hydraulic operating unit MO-22 (Narishige USA, Greenvale, NY) and a Nikon Diaphot™ inverted microscope equipped with a dry 40X objective (Nikon Inc., Rockville, MD). HeLa and PtK1 cells were grown on a coverslip attached over a hole in the bottom of a 60-mm Petri dish. In some experiments nocodazole and taxol were used at concentrations of 1.0 μM and 100 nM, respectively. The injection chamber was placed on the prewarmed microscope stage, and the cells were injected at different phases of cell division in groups of at least five cells per time point. Anti-survivin antibodies were introduced into the cells at concentrations 1.0–5.0 mg/ml diluted in KCl-PO_4 microinjection buffer. Control cells were injected with the KCl-PO_4 buffer. After microinjection, the cells were monitored and cinematographed by using a Hamamatsu CCD camera and image capture software (Hamamatsu Photonics Norden AB, Solna, Sweden). The images were processed further by using Adobe Photoshop (Adobe Systems Inc., San Jose, CA) and Corel Draw (Corel Corp., Ottawa, Ontario, Canada) software. In another set of independent experiments, HeLa cells were injected into the cytoplasm or nucleus with FITC-conjugated control or survivin antisense oligonucleotides at needle concentration of 10 μM. In each of four experiments, we injected 50–100 cells. Each injected cell was separated by at least 0.1 mm from another injected cell. The cells were cultured overnight, fixed with 3% paraformaldehyde in MBS (10 mM Mops; 150 mM NaCl, pH 7.4) followed by DNA-staining with DAPI (4,6-diamidino-2-phenylindole) for 2 min (200 ng/ml, Sigma) and examined by fluorescence microscope.

Analysis of the oligonucleotide transfected cells

In order to determine survivin protein levels after antisense targeting, we measured the fluorescence intensity of anti-survivin antibody detected survivin in cell populations treated with FITC-scrambled or FITC-survivin antisense oligonucleotides by using a GENIos plate reader (Tecan Inc., Research Triangle Park, NC). In brief, cells grown to ~60% confluence at the bottom of a 24-well plate were transfected with 400 nM scrambled control antisense, 200 nM survivin antisense, or 400 nM survivin antisense by using FuGENE 6 reagent (Roche Diagnostics Corporation). The cells were fixed 24 h later with 2.5% formaldehyde and were immunolabeled by using anti-survivin antibody detected with a Cy-3 secondary antibody. The fluorescence intensities were measured from two separate channels; Cy-3 channel was used to measure the fluorescence intensity of immunohistochemically detected survivin, while the fluorescein channel was used to measure the fluorescence intensity of the cells containing the FITC-conjugated antisense oligonucleotides. Three independent experiments, each containing three samples per an antisense oligonucleotide dilution, were conducted. Mock transfections (n=4) without any antisense oligonucleotides were used to determine the background fluorescence from the FITC- and Cy3-channels that was subtracted from the antisense targeted samples. To determine the effect of microinjected antisense oligonucleotides, we analyzed the morphology of a total of 200 HeLa cells in both control and survivin antisense oligonucleotide treated cell populations. We also scored the frequencies of micronucleated cells and cells showing signs of apoptosis (condensed chromatin and nuclear fragmentation) among a population of 1,500 cells from three individual experiments. Finally, we determined the percentages of M-phase cells showing signs of abortive mitosis (decondensation of chromatin and/or formation of cleavage furrow) after various incubation times in nocodazole (a total of 300 mitotic cells per each time point from three independent experiments were analyzed) within the control and survivin antisense oligonucleotide transfected PtK1 cell populations.

Immunofluorescence

For immunolabeling, cells growing on coverslips were simultaneously fixed and extracted for 15 min in 0.1% Triton X-100 in MBS containing 2.5% formaldehyde and 100 nM of phosphatase inhibitor microcystin LR. After washes with MBST (MBS with 0.05% Tween 20), the cells on were blocked with 20% boiled goat normal serum in MBST for 60 min. The cells were incubated in primary antibodies for 60 min. Cy3- or fluorescein-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) were used to visualize the primary antibody epitopes. Anti-rabbit IgG antibodies (Sigma, 1:800) were used to detect the injected anti-survivin antibodies. The DNA was counterstained with DAPI. For analysis of 3F3/2 phosphoepitopes in the anti-survivin and control buffer injected cells, we analyzed 5-to-10 cells per each time point (2–4 min, 6–8 min, 10–14, 16–20, and 22-28 min after nuclear envelope breakdown [NEB]). In the analyzed cells we calculated the number of 3F3/2 positive spots that co-localized with the kinetochores (Crest signals).

Statistical analysis

We performed statistical analysis by using either Student's t-test or two-factor ANOVA with a 95% confidence range.

RESULTS

Induction of abortive mitosis and polyploidy by survivin antisense oligonucleotides

To investigate the effects of inhibiting survivin during mitosis, we administrated FITC-conjugated control and survivin antisense oligonucleotides into the HeLa and PtK1 cells by either microinjection or transient transfection. In the HeLa cells transfected with the 2'-O-methoxyethyl chimaeric oligonucleotide at 200 and 400 nM concentrations, the endogenous survivin protein was reduced significantly in a concentration dependent manner by ~30% to 80% after survivin antisense targeting ($P<0.05$; 200 nM survivin antisense, $P<0.01$; 400 nM survivin antisense), whereas the scrambled control oligonucleotide had no effect (Fig. 1A). As the transfection efficiency varied between 30%–70% there was always a population of untransfected cells with normal amount of survivin RNA and protein present in the cell harvests. Thus it is likely that levels of survivin protein in the transfected cells were reduced even more. Earlier studies demonstrated induction of apoptosis on transfection with survivin antisense oligonucleotides (7, 8) usually ranging 10%–15% in cycling cell population. We did not observe any significant increase in the frequency of apoptotic cells after transfection with survivin antisense oligonucleotides, even after 24-h incubation. In both cell populations, a low percentage of apoptotic cell was scored (control, 1.3 ± 1.5 with range 1.1%–2.7%; survivin antisense, 2.2 ± 1.3, range 0.9%–3.3%). However, the frequencies of abnormally large cells and micronucleated cells were increased significantly in the survivin antisense oligonucleotide-transfected PtK1 cell populations compared with controls ($P<0.01$, Table 1). Induction of polyploidy by the survivin antisense oligonucleotides was confirmed with HeLa cells that were microinjected with either scrambled or survivin antisense oligonucleotides and were cultured overnight. In order to clearly determine that an injected cell progressed through M phase and formed two daughter cells, we microinjected cells that were apart from each other by at least 0.1 mm. In the population injected with FITC-conjugated survivin antisense oligonucleotides, 32.5% of the cells (65/200) did not divide but become abnormally large during the overnight incubation, whereas only 2% (4/200) of the control cells had the same phenotype (Fig. 1B, $P<0.01$). Next, we investigated the effect of microtubule drug-induced M phase block on the cells transfected with the control or survivin antisense oligonucleotides. In this experiment, we used PtK1 cells as they have certain advantages over the smaller HeLa cells; they have fewer chromosomes and remain flatter during M phase. Furthermore, micronuclei are more apparent in PtK1 cells compared with the HeLa cells. PtK1 cells were transfected with control or survivin antisense oligonucleotides at concentration of 400 nM, incubated overnight and were then arrested at M phase with the microtubule destabilizing drug nocodazole. The cells were then harvested at 0-, 2-, 4-, 6-, or 8-h time points. The frequency of fluorescent cells with micronuclei was significantly higher at 0-, 2-, 6-, ($P<0.05$), and 8-h ($P<0.01$) time points in the survivin antisense oligonucleotide-transfected cells compared with controls (Fig. 1C). We also determined how many nocodazole-treated M phase arrested cells showed signs of abortive mitosis, such as decondensation of chromatin and formation of cleavage furrow at the different time points (Fig. 1C). Survivin antisense-targeted mitotic cells aborted M phase more frequently than control cells especially at 6-h ($P<0.01$) and 8-h ($P<0.05$) time points where 19.0 ± 2.3% and 12.3 ± 3.5% were in process of exiting M phase, respectively. Cells in Figure 2 show the different phenotypes of the abortive mitosis

observed in the survivin antisense oligonucleotide-transfected PtK1 cells at the 6-h time point. From these results we conclude that antisense targeting of survivin induces cell division defects that lead to formation of polyploid and micronucleated cells. Moreover, cells lacking normal levels of survivin protein escape chemically induced M phase block and exit mitosis abnormally causing polyploidy and aneuploidy.

Anti-survivin antibodies induce premature onset of anaphase

In order to investigate the effects of perturbed survivin function during mitosis we used a microinjection technique. Many previous studies have relied solely on mutagenesis or antisense targeting of survivin. These strategies have resulted in a partial or total lack of functional survivin protein from the start of its normal expression at G2 phase of the cell cycle. By using anti-survivin antibody microinjection, we have been able to block function of survivin at specific mitotic phases in normal cells and in cells treated with microtubule-stabilizing or microtubule-destabilizing drugs. To evaluate the effects of anti-survivin antibodies (Alpha Diagnostics International Inc, San Antonio, TX, 1.0–5.0 mg/ml needle concentration in KCl-PO_4 microinjection buffer) on the progression of cell division, we first microinjected HeLa and PtK1 cells at different time points in mitosis from prophase through anaphase. We confirmed specificity of the antibody by Western blotting. In mitotic PtK1 cells a major band is detected at 16 kD (Fig. 3A). Immunofluorescent detection of anti-survivin antibody showed protein accumulation at the spindle poles (Fig. 3A). Only cells injected with anti-survivin antibodies between late prophase and early prometaphase showed a specific cell division defect. Essentially the same defect was observed in both HeLa and PtK1 cell lines. However, the flatter morphology and fewer chromosomes of the PtK1 cells allowed us to analyze mitosis in more detail by using live cell cinematography. Two cognate observations were made: anti-survivin antibody-injected PtK1 cells separated their sister chromatids prematurely at late prometaphase or cells spent a significantly shorter time at the metaphase stage. We found that 9 of 22 injected PtK1 cells (41%) initiated anaphase at late prometaphase before formation of metaphase plate. Figure 3B shows a representative set of micrographs from mitosis of a PtK1 cell injected at early prometaphase with 2.0 mg/ml of anti-survivin antibodies. Onset of anaphase occurs before all the chromosomes have moved to the spindle equator leading to uneven distribution of the sister chromatids between the two progeny cells. The remainder of the anti-survivin antibody-injected PtK1 cells (13 out of 22 cells, 59%) spent a significantly shorter time at the metaphase stage compared with the controls (Fig. 3C; 4.6 ± 1.1 min vs. 8.6 ± 1.9 min, $P<0.01$). Furthermore, the cells injected with anti-survivin antibodies at late prophase or at early prometaphase started anaphase in an average 26.8 ± 5.3 min (range 17-34 min) after the NEB, which is significantly different from the controls (Fig. 3C; 32.9 ± 6.9 min, $P<0.05$). All anti-survivin antibody-injected cells, including those experiencing premature onset of anaphase exit mitosis normally without defects in cytokinesis. We noted only a slight delay in anaphase and telophase chromosome movements and in progression of cytokinesis, possibly due to formation of chromosome bridges (Fig. 3C). Injection of metaphase ($n=5$) or anaphase ($n=5$) cells with anti-survivin antibodies had no effect on progression of late mitosis or exit from M phase (data not shown).

Anti-survivin antibody-injected cells escape the chemically induced M phase block

Spindle damage induced by microtubule poisons, such as nocodazole, is known to cause a prolonged metaphase arrest of several hours (13). To determine whether blocking of survivin function during mitotic arrest affects the length of the delay, we injected nocodazole- and taxol-treated PtK1 cells with anti-survivin antibodies. All of the anti-survivin antibody-injected cells (n=10) escaped the M phase block within 3 h of the injection (149.7 ± 37.8 min; Fig. 4), whereas all the control cells (n=10) remained arrested in M phase for at least 8 h (data not shown). Cells treated with spindle poisons and injected with anti-survivin antibodies decondensed their chromosomes and returned to interphase without completing cytokinesis and consequently formed micronucleated giant cells (Fig. 4). Similar results were observed with both taxol and nocodazole.

The 3F3/2 phosphoepitope is lost prematurely from the mitotic kinetochores of anti-survivin antibody-injected PtK1 cells

One marker for the spindle checkpoint is the 3F3/2 phosphoepitope that responds to physical tension mediated by the opposing spindle microtubules at the sister kinetochores of a chromosome (14, 15). In a normal cell, 3F3/2 epitopes are phosphorylated during prometaphase and localize to the kinetochores of chromosomes and to the spindle poles (14). As the chromosomes move to the spindle equator, the 3F3/2 epitope is dephosphorylated and is lost from the kinetochores but not from the spindle poles (14, 15). When all kinetochore-bound 3F3/2 is lost from each chromosome of a cell, the checkpoint is switched off and the cell cycle progresses to anaphase. To determine what happens to 3F3/2 phosphoepitope expression in the control buffer or anti-survivin antibody-injected PtK1 cells, we fixed and immunolabeled cells with anti-3F3/2 antibody at different time points after the manipulation of late prophase cells. Figure 5 shows that the 3F3/2 phosphoepitope is lost prematurely from the kinetochores of anti-survivin antibody-injected PtK1 cells. However, intensity of the 3F3/2 fluorescence signals at the spindle poles did not change over time in the anti-survivin antibody-injected cells (Fig. 5A). Rather, they stay at approximately the same level of intensity as in the control buffer-injected cells (data not shown). The kinetics of the loss of fluorescent 3F3/2 signals from the kinetochores corresponds well with the timing of the precocious separation of the sister chromatids. Loss of the kinetochore-bound 3F3/2 phosphoepitope from the anti-survivin antibody-injected cells takes in average 14.0 ± 3.0 min after NEB, which is significantly shorter compared with 24.0 ± 4.0 min of control buffer-injected cells (Fig 5B; P<0.01). After the loss of 3F3/2 phosphoepitope from the kinetochores there is a short period during which the inhibition of anaphase onset is alleviated. This same phenomenon has been reported earlier with nontreated cells (16) and was also observed here with the noninjected and control buffer-injected cells. In the control buffer-injected cells, the 3F3/2 phosphoepitope was lost normally from the kinetochores of an individual chromosome as it aligned at the spindle equator (Fig. 5B). Finally, we investigated whether introduction of anti-survivin antibodies affects spindle assembly. A population of PtK1 cells was injected at late prophase with anti-survivin antibodies or control buffer and was allowed to progress for 15 min in its division before fixation and immunolabeling with anti-tubulin antibodies. Analysis of the cells at the light microscope level demonstrates that

introduction of the anti-survivin antibody or control buffer does not induce any apparent spindle damage (data not shown).

DISCUSSION

The human survivin gene has been implicated in apoptosis and in the regulation of cell cycle, however, its exact functions remain controversial. Here we have investigated the mitotic role of survivin by using survivin antisense oligonucleotides and anti-survivin antibodies followed by fluorescence microscopy and live cell cinematography. We show, consistent with previous reports for human survivin (7, 8), that perturbation of the survivin function by antisense targeting at G2 phase of the cell cycle causes cell division defects that result in production of aneuploid and polyploid cells. In both HeLa and Ptk1 cells treated with survivin antisense oligonucleotides, the frequency of polyploidy was significantly higher compared with control cells treated with the scrambled oligonucleotides. However, we did not observe any significant increase in the number of apoptotic cells in the survivin antisense treated cells compared with the controls, contrary to earlier reports (7, 8). Probable explanations for this discrepancy are differences in the transfection procedures or in the timing and methodology of cell harvesting.

To investigate the possible mechanisms of induction of polyploidy in detail, we microinjected early mitotic PtK1 cells with anti-survivin antibodies and followed them as they progressed through mitosis. The injected anti-survivin antibody accumulates to the spindle poles. We were surprised that the anti-survivin antibody-injected cells exhibited premature separation of sister chromatids but had no apparent difficulties in exiting mitosis. In the anti-survivin antibody-injected cells, the duration of metaphase stage of mitosis was significantly shorter compared with controls. Furthermore, more than 40% of the anti-survivin antibody-injected cells separated their sister chromatids at late prometaphase before formation of a metaphase plate. To our knowledge, this is the first data suggesting a role for survivin in the actual process of chromosome segregation. Moreover, in the anti-survivin antibody-injected cells, the kinetochore-bound 3F3/2 phosphoepitope was lost prematurely at late prometaphase, which indicates that the spindle checkpoint was affected in these cells (15). Other evidence for the role of survivin in the regulation of mitosis came from studies conducted with cells that were chemically arrested at M phase. Normally, cells with a hyperactivated spindle checkpoint retain high cyclin B levels and keep the chromosomes condensed for at least 12 h. Introduction of survivin antisense oligonucleotides or anti-survivin antibodies into these cells induced abortive mitosis yielding aneuploidy and polyploidy. Together these results suggest a link between survivin function and control of the metaphase-anaphase transition by the spindle checkpoint.

A recent discovery of multiple human and murine survivin isoforms perhaps explains the different mitotic effects induced by survivin targeting (17). In both species, three differentially expressed survivin cDNAs with predicted isoform size of 140-, 120- and 40-amino acid residues have been identified, which suggests that survivin isoforms may play multiple roles in the regulation of apoptosis and cell division in mammals (Fig. 6). At this point, however, it is impossible to determine which survivin isoform(s) the approaches we have used target (although the anti-survivin antibody used recognizes only the full-length, 140-amino acid residue in the Western blots).

The present results also raise questions about the possible mitotic target(s) of survivin isoforms or the mitotic signaling cascade in which the proteins may participate (Fig. 6). Survivin shares a number of characteristics with classical M phase regulatory molecules. It transiently localizes to the kinetochores, centrosomes, and microtubules during mitosis (7, 11, 18), and it is destroyed by ubiquitin-mediated proteolysis in a cell cycle-dependent manner (19). Also, the survivin-like *C. elegans* BIR-1 protein interacts with aurora-like kinase AIR-2 (10). The members of aurora/Ipl1 kinase family have been implicated in cell division and chromosome segregation (20). For example, loss of function of *S. cerevisiae* Ipl1 results in aneuploidy and apoptosis (21), whereas overexpression of AIM-1 (Aurora and Ipl1-like midbody-associated protein-1) induces uneven distribution of chromosomes between the daughter cells and polyploidy (22). In addition, Aurora2 kinase associates with Cdc20 (23), a regulator of APC/C that counteracts effects of Mad2 (24), an inhibitor of APC/C activity (25). The binding of Cdc20 to APC/C and consequent loss of Mad2 from the complex activates the APC/C-mediated proteolysis of anaphase inhibitors and induces entry into anaphase (26, 27). Introducing anti-Mad2 antibodies into mitotic cells causes premature activation of APC/C and precocious onset of anaphase (28), an event similar to what we have observed after anti-survivin antibody injections. Given this, it is tempting to speculate that the cell division defects we detect after perturbation of survivin function could be caused by an effect on the function of the aurora/Ipl1 kinases or by an impact on the activity of the APC/C (Fig. 6). Finally, survivin may participate in regulation of the 3F3/2 phoshoepitope phosphorylation as the dephosphorylation and consequent loss of the 3F3/2 phophoepitope from the kinetochores at metaphase marks deactivation of the checkpoint and activation of the APC/C (29, 30), an event that occurred prematurely in anti-survivin antibody-injected cells. Together these results suggest that survivin is a mitotic regulator and possible component of the spindle checkpoint machinery. Elucidation of the mechanisms by which survivin modulates activity of the spindle checkpoint will be valuable in understanding how aneuploidy contributes to malignant cell growth and tumorigenesis.

Note added in proof

Wheatley et al. (Current Biology, 2001, vol. 11, 886-890) have recently shown that survivin associates directly to both aurora-B and INCENP in yeast two-hybrid and *in vitro* pull-down assays. We have also observed a metaphase delay in HeLa cells injected with another anti-survivin antibody that recognizes the kinetochore and spindle microtubule bound survivin (unpublished results).

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REFERENCES

1. Hardwick, K. G. (1998) The spindle checkpoint. *Trends Genet.* **14**, 1–4

2. Amon, A. (1999) The spindle checkpoint. *Curr. Opin. Genet. Dev.* **9**, 69–75

3. Gorbsky, G. J., Kallio, M., Daum, J. R., and Topper, L. M. (1999) Protein dynamics at the kinetochore: cell cycle regulation of the metaphase to anaphase transition. *FASEB J.* **13**, S231–234

4. Page, A. M., and Hieter, P. (1997) The anaphase promoting complex. *Cancer Surv.* **29**, 133–150

5. Peters, J. M. (1999) Subunits and substrates of the anaphase-promoting complex. *Exp. Cell Res.* **248**, 339–349

6. Li, F., Ambrosini, G., Chu, E. Y., Plescia, J., Tognin, S., Marchisio, P. C., and Altieri, D. C. (1998) Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* (*London*) **396**, 580–584

7. Li, F., Ackermann, E. J., Bennett, C. F., Rothermel, A. L., Plescia, J., Tognin, S., Villa, A., Marchisio, P. C., and Altieri, D. C. (1999) Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat. Cell Biol.* **1**, 461–466

8. Chen, J., Wu, W., Tahir, S. K., Kroeger, P. E., Rosenberg, S. H., Cowsert, L. M., Bennett, F., Krajewski, S., Krajewska, M., Welsh, K., Reed, J. C., and Ng, S. C. (2000) Down-regulation of survivin by antisense oligonucleotides increases apoptosis, inhibits cytokinesis and anchorage-independent growth. *Neoplasia* **2**, 235–241

9. Fraser, A. G., James, C., Evan, G. I., and Hengartner, M. O. (1999) Caenorhabditis elegans inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytokinesis. *Curr. Biol.* **9**, 292–301

10. Speliotes, E. K., Uren, A., Vaux, D., and Horvitz, H. R. (2000) The survivin-like C. elegans BIR-1 protein acts with the Aurora-like kinase AIR-2 to affect chromosomes and the spindle midzone. *Mol. Cell* **6**, 211–223

11. Uren, A. G., Wong, L., Pakusch, M., Fowler, K. J., Burrows, F. J., Vaux, D. L., and Choo, K. H. (2000) Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype. *Curr. Biol.* **10**, 1319–1328

12. O'Connor, D. S., Grossman, D., Plescia, J., Li, F., Zhang, H., Villa, A., Tognin, S., Marchisio, P. C., and Altieri, D. C. (2000) Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. *Proc. Natl. Acad. Sci. USA* **97**, 13103–13107

13. Jordan, M. A., Thrower, D., and Wilson, L. (1992) Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. *J. Cell Sci.* **102**, 401–416

14. Gorbsky, G. J. and Ricketts, W.A. (1993) Differential expression of a phosphoepitope at the kinetochores of moving chromosomes. *J. Cell Bio.* **122**, 1311–13121

15. Nicklas, R. B., Ward, S. C., and Gorbsky, G. J. (1995) Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint. *J. Cell Biol.* **130**, 929–939

16. Rieder, C. L., Schultz, A., Cole, R., and Sluder, G. (1994) Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J. Cell Biol.* **127**, 1301–1310

17. Conway, E. M., Pollefeyt, S., Cornelissen, J., DeBaere, I., Steiner-Mosonyi, M., Ong, K., Baens, M., Collen, D., and Schuh, A. C. (2000) Three differentially expressed survivin cDNA variants encode proteins with distinct antiapoptotic functions. *Blood* **95**, 1435–1442

18. Skoufias, D. A., Mollinari, C., Lacroix, F. B., and Margolis, R. L. (2000) Human survivin is a kinetochore-associated passenger protein. *J. Cell Biol.* **151**, 1575–1582

19. Zhao, J., Tenev, T., Martins, L. M., Downward, J., and Lemoine, N. R. (2000) The ubiquitin-proteasome pathway regulates survivin degradation in a cell cycle-dependent manner. *J. Cell Science* **113**, 4363–4371

20. Bischoff, J. R. and Plowman, G. D. (1999) The Aurora/Ipl1p kinase family: regulators of chromosome segregation and cytokinesis. *Trends Cell Biol.* **11**, 454–459

21. Francisco, L., Wang, W., and Chan, C. S. (1994) Type 1 protein phosphatase acts in opposition to IpL1 protein kinase in regulating yeast chromosome segregation. *Mol. Cell Biol.* **14**, 4731–4740

22. Tatsuka, M., Katayama, H., Ota, T., Tanaka, T., Odashima, S., Suzuki, F., and Terada, Y. (1998) Multinuclearity and increased ploidy caused by overexpression of the aurora- and Ipl1-like midbody-associated protein mitotic kinase in human cancer cells. *Cancer Res.* **58**, 4811–4816

23. Farruggio, D. C., Townsley, F. M., and Ruderman, J. V. (1999) Cdc20 associates with the kinase aurora2/Aik. *Proc. Natl. Acad. Sci. USA* **96**, 7306–7311

24. Fang, G., Yu, H., and Kirschner, M. W. (1998) Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. *Mol. Cell* **2**, 163–171

25. Li, Y., Gorbea, C., Mahaffey, D., Rechsteiner, M., and Benezra, R. (1997) MAD2 associates with the cyclosome/anaphase-promoting complex and inhibits its activity. *Proc. Natl. Acad. Sci. USA* **94**, 12431–1246

26. Kallio, M., Weinstein, J., Daum, J. R., Burke, D. J., and Gorbsky, G. J. (1998) Mammalian p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events. *J. Cell Biol.* **141**, 1393–1406

27. Fang, G., Yu, H., and Kirschner, M. W. (1998) The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev.* **12**, 1871–1883

28. Gorbsky, G. J., Chen, R. H., and Murray, A. W. (1998) Microinjection of antibody to Mad2 protein into mammalian cells in mitosis induces premature anaphase. *J. Cell Biol.* **141**, 1193–1205

29. Campbell, M. S. and Gorbsky, G. J. (1995) Microinjection of mitotic cells with the 3F3/2 anti-phosphoepitope antibody delays the onset of anaphase. *J. Cell Biol.* **129**, 1195–1204

30. Daum, J. R., Tugendreich, S., Topper, L. M., Jorgensen, P. M., Hoog, C., Hieter, P., and Gorbsky, G. J. (2000) The 3F3/2 anti-phosphoepitope antibody binds the mitotically phosphorylated anaphase-promoting complex/cyclosome. *Curr. Biol.* **10**, R850–852

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Table 1

Induction of polyploidy and micronuclei by survivin antisense oligonucleotides in PtK1 cells

	scrambled antisense (400 nM)	survivin antisense (400 nM)
polyploid cells (%)	47/1500 (3.1 ± 0.8)	272/1500 (18.1 ± 3.3)*
MN (%)	2.5 ± 1.5	11.5 ± 3.5*

*Significantly different from the control ($P<0.01$).

Fig. 1

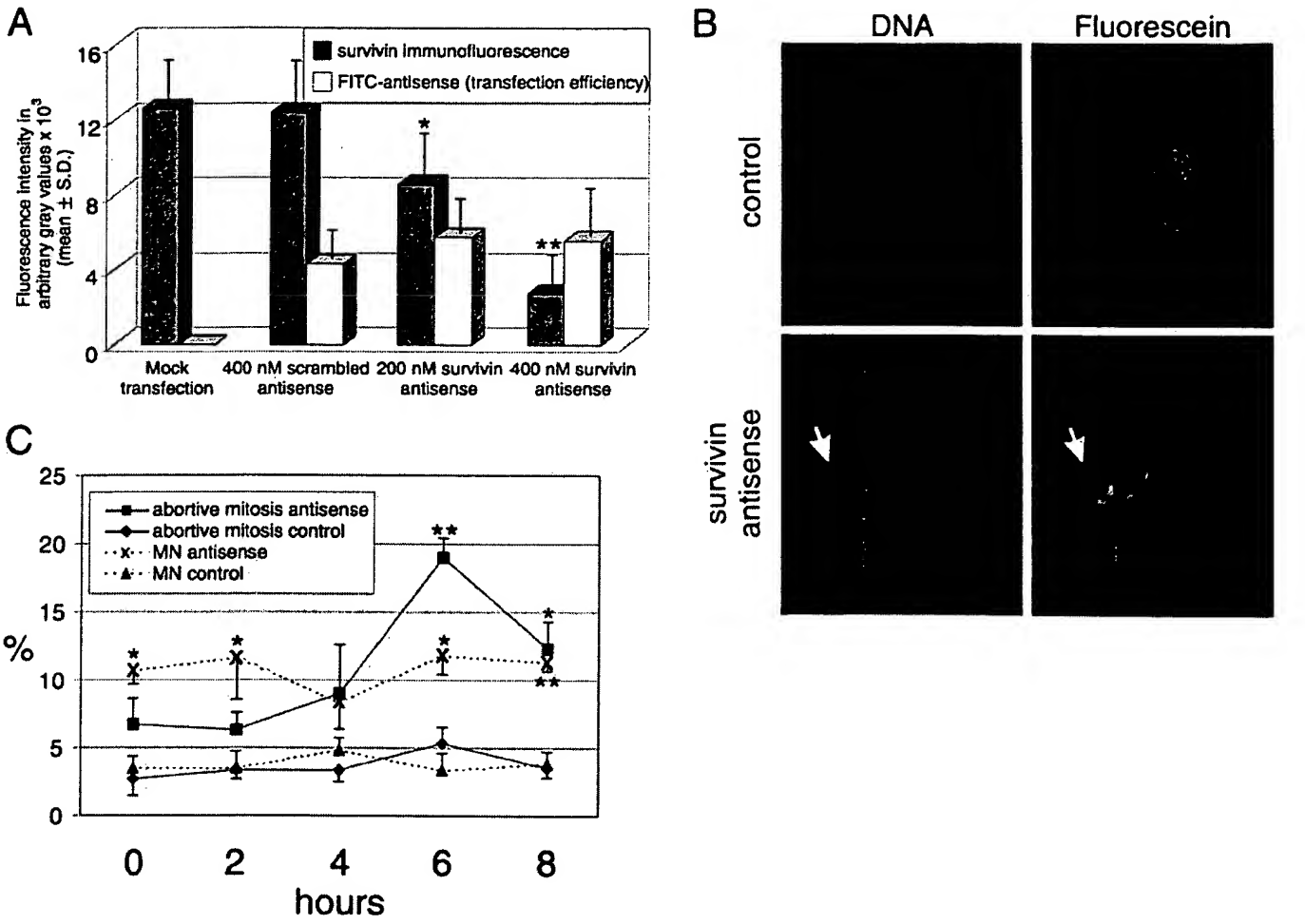

Figure 1. Induction of polyploidy and micronuclei after survivin antisense targeting. A) Survivin protein levels were reduced significantly in HeLa cells in a concentration-dependent manner after transfection with survivin antisense oligonucleotides. Fluorescence intensities of immunohistochemically labeled survivin protein and FITC conjugated scrambled and survivin antisense oligonucleotides were measured with a GENIos plate reader by using two separate channels for Cy3 and FITC. The transfection efficiency, based on the FITC-channel fluorescence, did not differ markedly between the scrambled and survivin antisense oligonucleotide-transfected samples. The asterisks denote statistical difference (*P<0.05; **P<0.01) in survivin protein levels between the scrambled antisense and survivin antisense targeted cell populations. **B)** HeLa cells were microinjected with FITC-conjugated scrambled antisense or survivin antisense oligonucleotides at a needle concentration of 10 μM and then incubated overnight. Many cells injected with the survivin antisense oligonucleotides had formed a large polyploid cell, some with MN (arrowhead), whereas most of the control cells had progressed through apparently normal mitosis and had formed two progeny cells of equal size. **C)** Induction of micronuclei and abortive mitosis after survivin antisense targeting of nocodazole treated PtK1 cells. Cells transfected with FITC-conjugated control or survivin antisense oligonucleotides at concentration of 400 nM were treated with nocodazole (1.0 μg/ml) for indicated time periods and then were analyzed for induction of micronuclei and abortive mitosis. Significantly more micronuclei were observed in the survivin antisense-targeted cells than in the controls at 0-, 2-, 6-, and 8-h time points. Many of the survivin antisense targeted cells were exiting the M phase especially at 6- and 8-h time points.

Fig. 2

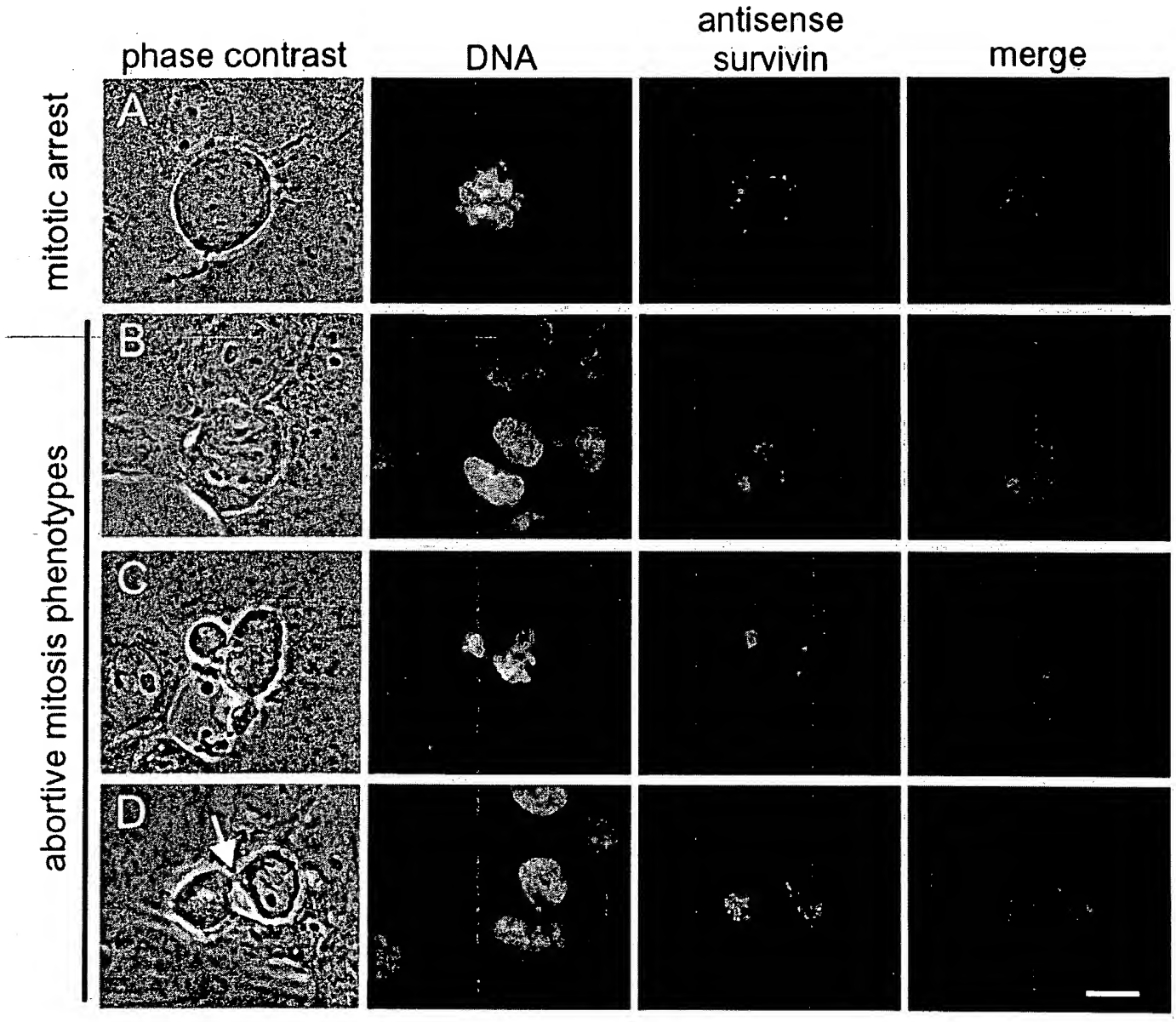

Figure 2. Morphology of PtK1 cells undergoing abortive mitosis after survivin antisense targeting and nocodazole treatment. The left panel shows the phase contrast image, the middle panels show the FITC and DAPI channels, respectively, and the right-hand panel shows the merge of FITC-conjugated survivin antisense oligonucleotides and DNA. After 6 h in nocodazole, 81% of the mitotic fluorescent cells remained at M-phase arrest with condensed chromosomes **A)** However, 19% of the FITC-positive cells were in the process of aborting mitosis **(B–D). B)** Some cells had already exited the M phase and had formed binuclear interphase cells indicative of errors in cytokinesis. **C)** Most of the cells aborting the M phase had misplaced cleavage furrows, which resulted in uneven distribution of chromosomes between the progeny cells. **D)** Occasionally polyploid early G1 cells were observed. The arrow points to the midbody between the daughter cells. Bar equals to 10 μm.

Fig. 3

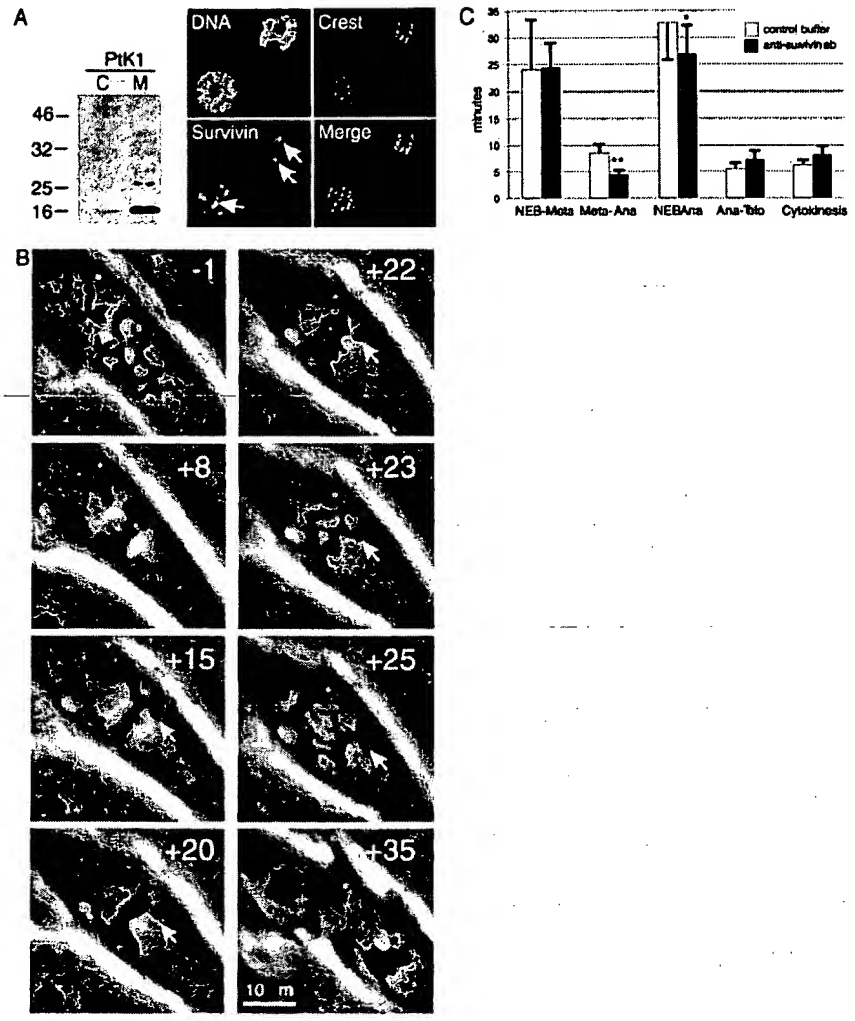

Figure 3. Effects of anti-survivin antibody microinjection on progression of cell division. A) Western blotting with anti-survivin antibody (C, cycling cell extract; M, mitotic cell extract). In the mitotic PtK1 cell extracts, there is a major band at ~16 kD, although a slight reactivity is seen with additional band at 26 kD. The microscope panels show the immunofluorescent localization of the survivin (red in the merge image) and kinetochores (Crest, green) in mitotic PtK1 cells. Some of the protein accumulates to the spindle poles during late prophase (arrowheads), whereas the rest of the protein remains diffuse in the cytoplasm. DNA is stained with DAPI (blue). **B)** Time-lapse sequence of a PtK1 cell injected with 2.0 mg/ml of anti-survivin antibody 2 min after NEB (injection is time point 0 min). The chromosomes move towards the spindle equator, and 15 min after the injection all chromosomes, except one (arrowhead), have aligned at the equatorial plate. When the sister chromatids of aligned chromosomes separate 22 min after the injection, the unaligned chromosome still appears to be mono-oriented as both of its sister chromatids move to the same spindle pole (arrowheads), resulting in uneven distribution of genetic material between the daughter cells. **C)** Diagram showing the duration of different mitotic phases in control buffer and anti-survivin antibody-injected PtK1 cells. The anti-survivin antibody injection did not affect the prometaphase chromosome movements, as the mean duration of the prometaphase (NEB-Meta) in anti-survivin-injected cells was near the average duration of prometaphase in control buffer-injected cells. However, both the average duration of metaphase stage (Meta-Ana, this group, n=13, includes only those anti-survivin antibody-injected cells that formed a metaphase plate and did not experience onset of anaphase in presence of unaligned chromosomes) and the average time from NEB to onset of anaphase were significantly shorter in the anti-survivin antibody-injected cells compared with controls. A slightly longer mean duration of anaphase phase and cytokinesis was observed in the anti-survivin antibody-injected cells compared with controls, which is possibly due to extra time needed to resolve the chromosome bridges in the anti-survivin antibody-injected cells.

Fig. 4

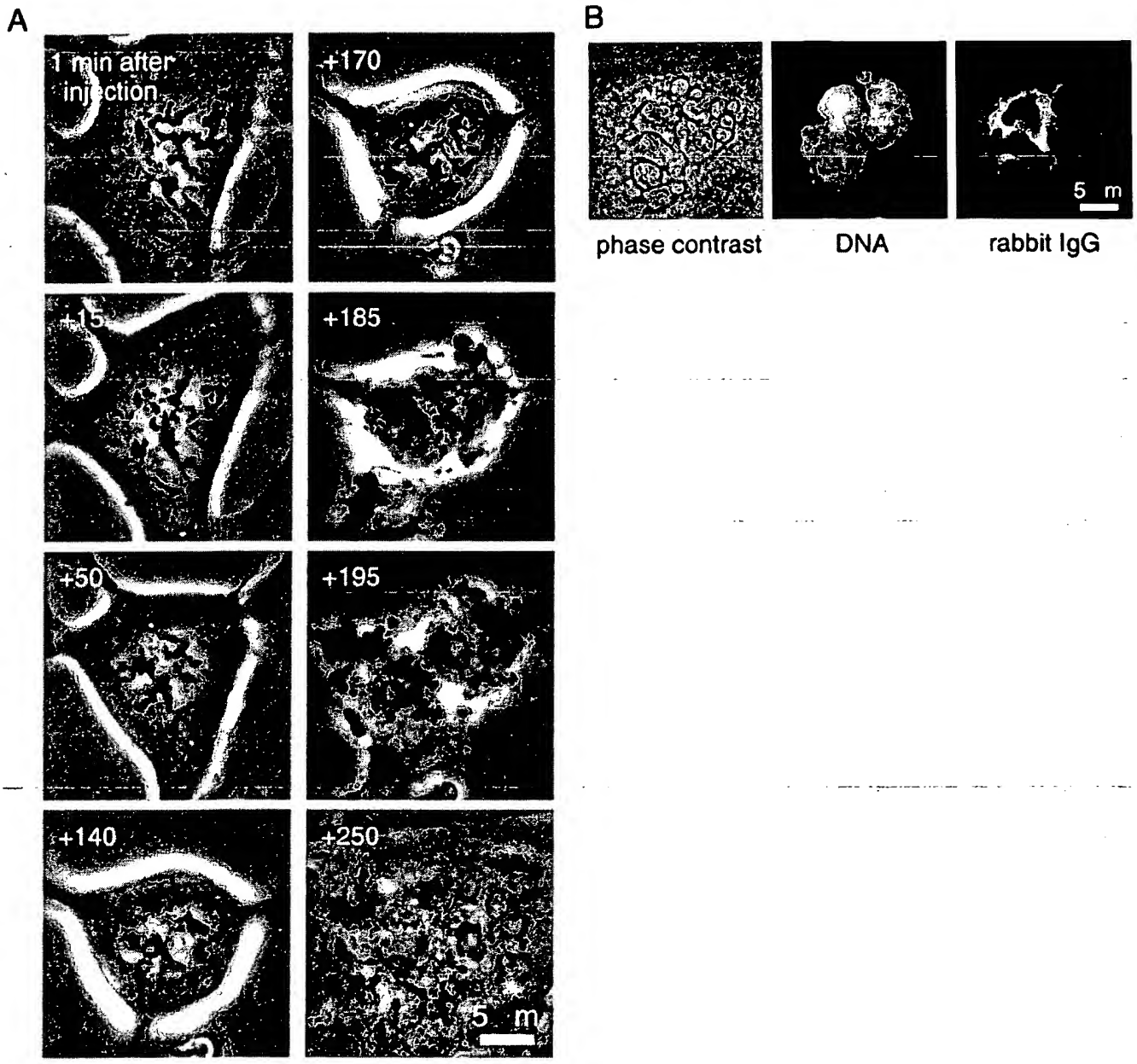

Figure 4. Anti-survivin antibody-injected PtK1 cells escape the chemically induced M phase arrest. PtK1 cells were arrested at M phase with nocodazole or taxol 30 to 60 min before injection either with anti-survivin or control buffer (n=5 for both drugs and control group). **A)** Time-lapse sequence of a mitotic PtK1 cell arrested at mitosis with nocodazole and then injected with anti-survivin antibody (2.0 mg/ml). This cell was treated with the drug for 30 min before introduction of the antibody. Approximately 170 min after the injection, the sister chromatids were separated slightly. Soon after, the cell membrane started rigorous blebbing, the chromosomes decondensed (time points +175–210 min), and the cell exited M phase. **B)** The same cell was fixed and immunolabeled with anti-rabbit IgG to visualize the injected anti-survivin antibody and DAPI stained for DNA. The cell had not completed cytokinesis, instead it had formed a giant polyploid cell with a fragmented nucleus.

Fig. 5

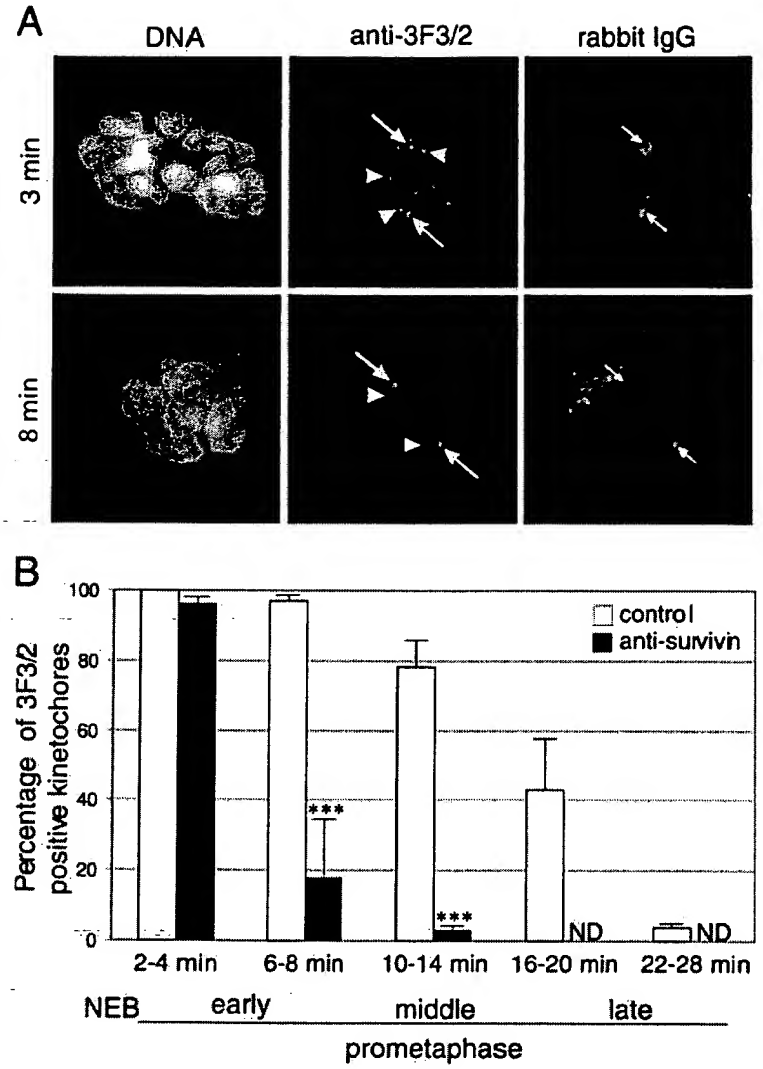

Figure 5. 3F3/2 phosphoepitope is lost precociously from the kinetochores of mitotic PtK1 cells after introduction of anti-survivin antibody. A) Fluorescent micrographs depicting two PtK1 cells that were injected with anti-survivin antibody (3.0 mg/ml) at early prometaphase. The cells were allowed to progress in their division for 3 and 8 min, respectively, before fixation and immunolabeling with anti-3F3/2 ascites and anti-rabbit IgG. The cell fixed 3 min after injection had a normal 3F3/2-staining pattern with bright signals at the kinetochores of unaligned chromosomes (some depicted with arrowheads) and spindle poles (arrows). The cell fixed 8 min after the injection of had lost most of its kinetochore-bound 3F3/2 epitope, although the cell was still at prometaphase stage with many unaligned chromosomes. Only two faint 3F3/2-positive kinetochores could be seen (arrowheads). The spindle poles had normal 3F3/2 signals (arrows). In both injected cells, the anti-survivin antibody accumulated at the spindle poles (see the arrows in the rabbit IgG images). **B)** Percentage of 3F3/2 positive kinetochores in cell populations injected with anti-survivin antibody or control buffer. Groups of 5 to 10 PtK1 cells were injected with the antibody at late prophase or early prometaphase and allowed to progress in their division for the times indicated before fixation and immunolabeling with anti-3F3/2 ascites and Crest anti-centromere sera. The percentage of 3F3/2 spots colocalizing with Crest signals was determined for each injected cell. All the kinetochore-bound 3F3/2 was lost within 15 min of the anti-survivin injection; even the cells were at prometaphase with some unaligned chromosomes. In the control cells, the 3F3/2 epitope remained at the kinetochores of unaligned chromosomes until the chromosomes moved to the spindle equator. The asterisks denote statistical difference between the control and anti-survivin antibody-injected cell populations at specific time points ($P<0.01$; ND, not detected).

Fig. 6

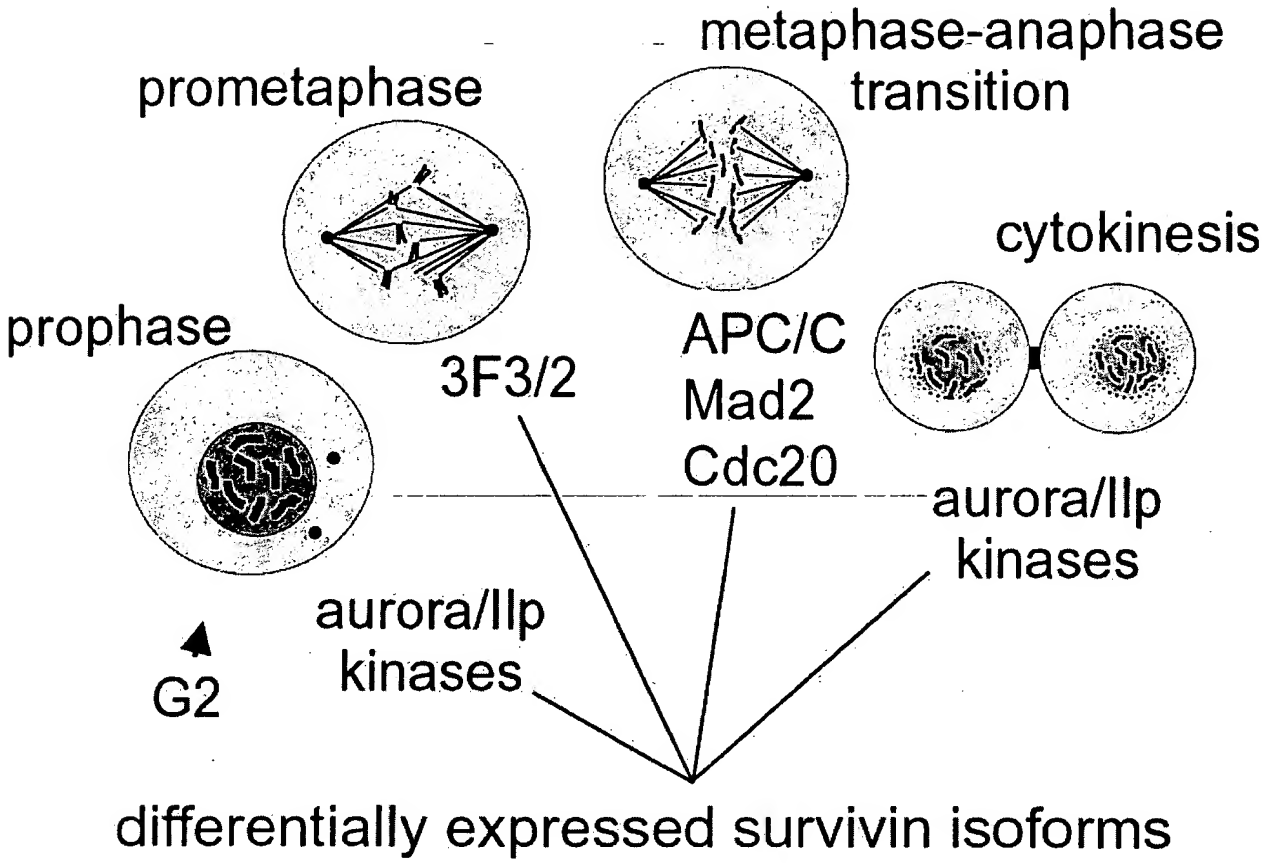

Figure 6. Possible targets of survivin isoforms during mitosis. The differentially expressed survivin isoforms may participate in regulation of various cell division events, including separation of sister chromatids and cytokinesis. See discussion for details.